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Effects of Modified Phycobilin Biosynthesis in the Cyanobacterium *Synechococcus* sp. Strain PCC 7002[▽]

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The pathway for phycocyanobilin biosynthesis in *Synechococcus* sp. strain PCC 7002 comprises two enzymes: heme oxygenase and phycocyanobilin synthase (PcyA). The phycobilin content of cells can be modified by overexpressing genes encoding alternative enzymes for biliverdin reduction. Overexpression of the *pebAB* and *HY2* genes, encoding alternative ferredoxin-dependent biliverdin reductases, caused unique effects due to the overproduction of phycoerythrobilin and phytochromobilin, respectively. Colonies overexpressing *pebAB* became reddish brown and visually resembled strains that naturally produce phycoerythrin. This was almost exclusively due to the replacement of phycocyanobilin by phycoerythrobilin on the phycocyanin α -subunit. This phenotype was unstable, and such strains rapidly reverted to the wild-type appearance, presumably due to strong selective pressure to inactivate *pebAB* expression. Overproduction of phytochromobilin, synthesized by the *Arabidopsis thaliana* *HY2* product, was tolerated much better. Cells overexpressing *HY2* were only slightly less pigmented and blue-green than the wild type. Although the *pcyA* gene could not be inactivated in the wild type, *pcyA* was easily inactivated when cells expressed *HY2*. These results indicate that phytochromobilin can functionally substitute for phycocyanobilin in *Synechococcus* sp. strain PCC 7002. Although functional phycobilisomes were assembled in this strain, the overall phycobiliprotein content of cells was lower, the efficiency of energy transfer by these phycobilisomes was lower than for wild-type phycobilisomes, and the absorption cross-section of the cells was reduced relative to that of the wild type because of an increased spectral overlap of the modified phycobiliproteins with chlorophyll *a*. As a result, the strain producing phycobiliproteins carrying phytochromobilin grew much more slowly at low light intensity.

Most cyanobacteria employ light-harvesting antennae known as phycobilisomes (PBS) to collect light that is not efficiently absorbed by chlorophyll (Chl) for photosynthesis. PBS are multisubunit, supramolecular structures composed of both pigmented phycobiliproteins (PBPs) and usually nonpigmented linker proteins (14). Four different linear tetrapyrrole chromophores (bilins), phycocyanobilin (PCB), phycoerythrobilin (PEB), phycoviolobilin (PVB), and phycourobilin (PUB), can naturally be bound to cyanobacterial PBPs (28). These four bilins are isomers that differ only in the number of conjugated double bonds that form the chromophore, and all are derived from a common biosynthetic precursor, biliverdin IX α (2–4, 8, 19, 49). Biliverdin IX α is synthesized from heme by oxidative cleavage of the α -methine bridge of heme by the enzyme heme oxygenase (11). PCB:ferredoxin oxidoreductase, PcyA, uses four electrons from reduced ferredoxin to synthesize PCB by regio-specific reduction of the *exo* vinyl group of ring D and the *endo* vinyl group of ring A of biliverdin IX α (19). The reactions leading to the conversion of heme into biliverdin IX α , PCB, and PEB are shown in Fig. 1. PEB is synthesized in a similar manner from biliverdin IX α by the sequential actions of two reductases, *PebA* and *PebB*, or the more recently discovered cyanoviral enzyme *PebS*, which catalyzes the same reactions performed jointly by *PebA* and *PebB* (Fig. 1) (12, 19). The

remaining two chromophores, PUB and PVB, are synthesized from PEB and PCB, respectively, by isomerizing lyases that both isomerize the precursor chromophores and attach them to their cognate PBP subunits (8, 49).

Various species of cyanobacteria utilize different combinations of chromophores and PBPs to optimize their light-harvesting capabilities for photosynthesis. This is generally believed to be due to adaptations to the specific light conditions available to a given species in its natural environment. As suggested by the phylum name, the archetypical cyanobacterium is blue-green and synthesizes Chl *a* and PBS containing only the blue (orange-absorbing) phycocyanin (PC) and aqua (red-absorbing) allophycocyanin (AP), both of which only carry PCB chromophores. A few species of cyanobacteria can synthesize phycoerythrocyanin, a fuchsia-colored protein whose α -subunit (*PecA*) carries a single PVB chromophore (10). Still other cyanobacteria, many of which are marine organisms, can produce phycoerythrin (PE), a red (green-absorbing) protein that carries PEB and sometimes PUB chromophores. Thus, proteins incorporating PCB have the most red-shifted absorption maxima, while those with PUB have the most blue-shifted maxima. Many cyanobacteria also possess the ability to adjust their spectral profile to absorb optimally the light available in their environment. Some PE-producing species do this by coordinately regulating apoproteins, lyases, and chromophore production, as in type II or type III complementary chromatic acclimation (25). Other species, such as the type IV chromatically acclimating species, adjust their spectral profiles to their light environment by simply regulating specific lyases

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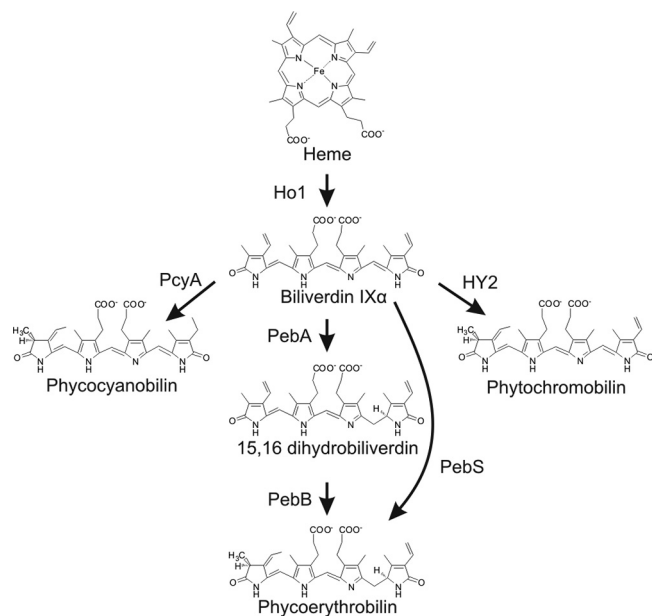


FIG. 1. Scheme showing the synthesis of linear tetrapyrrole chromophores in cyanobacteria, algae, and plants.

to produce an isomerizing or nonisomerizing lyase, which then dictates the conjugation state, and thus the absorption properties, of an attached chromophore (16).

Phytochromobilin (PΦB), the chromophore of higher-plant photosensory proteins known as phytochromes, is similar in structure to PCB, but no cyanobacterium has been shown to synthesize this bilin naturally. PΦB shares the same biliverdin IX α precursor as PCB and PEB, is more oxidized than PCB, and only differs from PCB by the presence of a vinyl group at the C-18 position (Fig. 1) (19). HY2, the ferredoxin-dependent biliverdin reductase responsible for PΦB biosynthesis from biliverdin in plants, does not occur naturally in cyanobacteria.

Properly assembled PBPs are important to cyanobacterial light harvesting for growth. Because of the variety of possible chromophores that may be found in a particular cell type, it has largely been assumed that the bilin lyases responsible for the attachment of chromophores to their cognate apoprotein must exhibit a high degree of specificity in the recognition of their appropriate bilin chromophore and its acceptor apoprotein. PBP lyases have been most completely defined in the cyanobacterium *Synechococcus* sp. PCC 7002 (34). The CpcE/CpcF heterodimeric lyase is solely responsible for the attachment of PCB to CpcA (51). CpcT attaches PCB to the Cys153 site on CpcB (35), while the CpcS/CpcU heterodimer attaches PCB to the remaining site on CpcB (Cys82) and to the single chromophore-binding sites on ApcA, ApcB, ApcD, and ApcF (7, 33, 36). Cyanobacterial species that also produce PE are thought to have additional PEB-specific lyases that catalyze chromophore attachment to these proteins, but these lyases have remained largely uncharacterized (23, 46, 50).

The studies here report the effects of genetic alterations of chromophore content on PBPs through the overexpression of HY2 and *pebAB* and the insertional inactivation of *pcyA* in a

cyanobacterial strain that naturally possesses only *pcyA* as its sole ferredoxin-dependent biliverdin reductase.

MATERIALS AND METHODS

Cyanobacterial growth conditions. Medium A⁺ (medium A containing 12 mM sodium nitrate) was routinely used for growth of both wild-type (WT) and mutant strains of *Synechococcus* sp. strain PCC 7002 (40). Unless otherwise specified, cells were grown under standard conditions, which are 38°C, 250 μ mol photons m⁻² s⁻¹, and with sparging with air supplemented with 1% (vol/vol) CO₂. A glycerol-tolerant strain of *Synechococcus* sp. strain PCC 7002 was generated through the continuous growth of the wild-type strain in liquid A⁺ medium containing 10 mM glycerol and was used as a background for the generation of the *pebAB* and HY2 overexpression strains (20). Spectinomycin at 50 μ g ml⁻¹ and gentamicin at 50 μ g ml⁻¹ were added to media as needed for selection and maintenance of transformants (20). Before, during, and after transformations, cells were cultivated at approximately 200 μ mol photons m⁻² s⁻¹ of cool white fluorescent light.

Construction of bilin biosynthesis mutants. To inactivate the *pcyA* gene of *Synechococcus* sp. strain PCC 7002, regions of approximately 600 bp immediately upstream and downstream of the *pcyA* gene were amplified by PCR with primers pcyAR1F (5'-GCACTGATGCATATTCTGTGTGGACATCGTAGC-3'; NsiI site underlined) and pcyAR1R (5'-GGGCAGCCATGGGCGAAAAAGCAATCTTA A-3'; NcoI site underlined) for the upstream sequence and pcyAR2F (5'-TATTC CCCATGGGTTCGACGAACAAAGCTTTAATTACCGCAG-3'; NcoI and SalI sites underlined) and pcyAR2R (5'-ACCTGAGCATGCTTCAGCCGACCCCTA CCGA-3'; SphI site underlined) for the downstream sequence. These fragments were digested with NcoI and ligated together. The resulting product was digested with SphI and NsiI and ligated into pGEM-7Zf(+) (Promega Corporation, Madison, WI) to make pGEM-*pcyAR1R2*. The *aadA* cassette from pSRA81 (20) was amplified by PCR with primers pst-aadAF (5'-GGTGCTCCATGGGGCTGCTAA CAAAGCCCGAAA-3'; NcoI site underlined) and sal-aadAR (5'-GGAGGCGTC GACCTAGAGTCGAGCGAATTGTTAG-3'; SalI site underlined), and the resulting product was digested with NcoI and SalI and cloned into similarly digested pGEM-*pcyAR1R2*. The resulting plasmid was used as a template in a PCR using primers pcyAR1F and pcyAR2R to create a 2.3-kb linear DNA fragment for natural transformation of a glycerol-adapted strain of *Synechococcus* sp. strain PCC 7002. The primers pcyAR1F and pcyAR2R were also used to assess segregation of the *pcyA* and *pcyA::aadA* alleles at the *pcyA* locus.

To introduce *pebAB* genes into plasmid pAQ1 of *Synechococcus* sp. strain PCC 7002, the *pebAB* genes from *Synechococcus* sp. strain PCC 7335 were amplified by PCR with primers 7335pebAF (5'-GCTGTGTGATATGATCGCCCTTTT CAAGCG-3'; NdeI site underlined) and 7335pebBR (5'-ATATTGAGGATC CTTAGGCGAGCGATGCGAGCG-3'; BamHI site underlined). The resulting amplicon was digested with NdeI and BamHI and cloned into similarly digested pAQ1cpcEx (47). This plasmid allows for the convenient cloning and introduction of foreign genes, the expression of which is driven by the *cpcBA* promoter of *Synechocystis* sp. strain PCC 6803, into the endogenous pAQ1 plasmid of *Synechococcus* sp. strain PCC 7002 by homologous recombination.

The HY2 gene was also expressed on endogenous plasmid pAQ1 under the control of the *Synechocystis* sp. strain PCC 6803 *cpcBA* promoter; however, due to the presence of the most convenient restriction sites (in particular NdeI, NcoI, and BamHI) in the HY2 gene itself, the pAQ1cpcEx vector was modified to facilitate the cloning of this gene. First, the gentamicin cassette from pMS266 (5) was amplified using primers gentF (5'-ATCCGTTCTAGAAGGTCTTCCGATCTCCTGAAG-3'; XbaI site underlined) and gentR (5'-CCTAGAGTCGACGTCGCCGGGAA GCCGAT-3'; SalI site underlined). The resulting product was digested with XbaI and SalI and cloned into pAQ1cpcEx that had also been digested with XbaI and SalI. This switched the drug resistance cartridge from *aadA*, which confers spectinomycin resistance, to *aacCl*, which confers gentamicin resistance. In order to replace the BamHI site on this vector with PstI, a region including this new cassette was amplified by PCR with primers pAQ1cpcEx (5'-GGTGCTCCATGGGCTGCAGGG CTGCTAACAAAGCCCGAAA-3'; NcoI and PstI sites underlined) and gentR. This product was then digested with NcoI and SalI and cloned into pAQ1cpcEx that had been similarly digested. The HY2 gene was amplified by PCR from plasmid pPL-PΦB (18) by using the primers bspHY2F (5'-CCCTGGTCATGATCTCTGC TGTGTCGTATAAGG-3'; BspHI site underlined) and KOHY2R (5'-TATGCTG CAGTTAGCCGATAAATTGCTGTAAATCCCAAGGACGAG-3'; PstI site underlined), cut with BspHI and PstI, and cloned into this modified pAQ1cpcEx vector (47), which was cut with NcoI and PstI.

To create linear DNA fragments for natural transformation into *Synechococcus* sp. strain PCC 7002, the *pebAB* and HY2 plasmids were used as templates in PCRs with the primers pAQ1sph (5'-CCCGACGTCGCATGCCTC-3') and pAQ1nsi (5'-GAATACTCAAGCTATGCATGGG-3').

Spectroscopic and compositional analyses of bilin biosynthesis strains and their PBS. Absorption spectra of whole cells of *Synechococcus* sp. strain PCC 7002 and its isolated PBS were recorded with a GENESYS 10 spectrophotometer (ThermoSpectronic, Rochester, NY).

Chl *a* was extracted using 100% methanol, and concentrations were determined according to previously described methods (27, 29). The relative PBP contents of cells were determined using heat-induced bleaching at 65°C as described previously (32, 48). Because the P Φ B chromophore has a more red-shifted absorption than the PCB chromophore, the method was modified slightly; the peak absorption maxima differences between the PBPs of the two strains were compared.

PBSs were isolated as described previously (35), and their protein contents were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). Proteins separated on a 14% (wt/vol) polyacrylamide gel were first stained with a 100 mM ZnCl₂ solution to enhance the fluorescence of the bilins (6), which were visualized using a Typhoon 8600 variable mode imager (GE Healthcare Life Sciences, Pittsburgh, PA). Total proteins were then stained with Coomassie blue and imaged with an Epson Perfection V750 flatbed scanner. His-tagged preparations of *Synechocystis* sp. strain PCC 6803 holo-CpcA, chromophorylated with either PCB or PEB, were used for comparisons in these experiments. These recombinant proteins were heterologously expressed in *Escherichia coli* BL21(DE3) cotransformed with pBS414v (43) and either pPcyA (7) for PCB-CpcA or pTDho1pebS (12) for PEB-CpcA and were isolated by metal chelation chromatography as described previously (43).

Fluorescence measurements of whole cells and isolated PBS were recorded with an SLM 8000C spectrofluorometer that was modernized for computerized data acquisition by On-Line Systems, Inc. (Bogart, GA). Whole-cell fluorescence at 77 K was measured by resuspending cells in 50 mM HEPES-NaOH buffer, pH 7.0, containing 60% (vol/vol) glycerol, and the mixtures were frozen in liquid nitrogen as described previously (35).

PBPs were analyzed by a high-performance liquid chromatography (HPLC) method similar to that previously described (41). Prior to HPLC separation, PBP samples were dialyzed against 10 mM sodium phosphate (pH 7.0), 1.0 mM 2-mercaptoethanol. The dialyzed protein solutions were diluted 1:1 (vol/vol) with 6 M guanidinium hydrochloride, pH 5.2, and centrifuged for 5 min prior to injection onto a C₄ reversed-phase HPLC column (RP-304; 4.5 mm by 350 mm; Bio-Rad, Richmond, CA) that had previously been equilibrated with 65% trifluoroacetic acid (TFA; 0.1%) in water (buffer A), and 35% 2:1 acetonitrile-isopropanol containing 0.1% TFA (buffer B). Proteins were eluted from the column at a flow rate of 1.5 ml min⁻¹ according to the following program: 0 to 3 min, 65% buffer A–35% buffer B; 3 to 37 min, linear gradient to 30% buffer A–70% buffer B; 37 to 45 min, linear gradient to 100% buffer B; 45 to 55 min, linear gradient to 65% buffer A–35% buffer B. HPLC was performed using Waters model 510 pumps and a model 600 automated gradient controller (Waters Chromatography Division, Milford, MA). Data were acquired using a Waters 2996 photodiode array detector; spectra were collected from 220 to 700 nm at 1-s intervals.

RESULTS

Attempted insertional inactivation of *pcyA* in wild-type *Synechococcus* sp. strain PCC 7002. In order to gain a better understanding of the dual role of PCB in both light harvesting and light sensing in *Synechococcus* sp. strain PCC 7002, an attempt was made to produce a *pcyA* null mutant by insertional inactivation of the gene. As for other mutations that could potentially affect the photosynthetic capacity of this cyanobacterium, constructions to inactivate the *pcyA* gene were transformed into a glycerol-tolerant strain that could grow photo-mixotrophically on glycerol (20). Similar conditions were previously employed to produce mutants lacking PC and AP in this cyanobacterium (9). Colonies from the resulting transformation with the *pcyA::aadA* construct were initially indistinguishable from wild-type *Synechococcus* sp. strain PCC 7002 (Fig. 2A). However, upon several rounds of streaking and selection, the majority of the colonies were yellow-green (chlorotic), which was consistent with the inability to synthesize

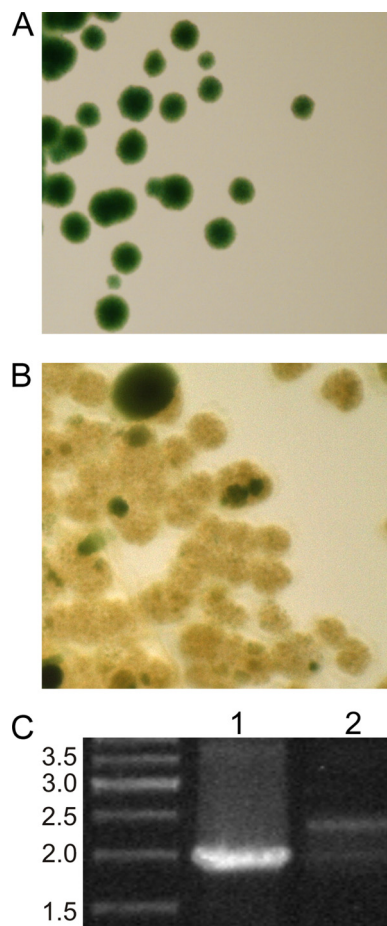


FIG. 2. The *pcyA* gene is apparently essential in *Synechococcus* sp. strain PCC 7002. (A) Colonies of wild-type *Synechococcus* sp. strain PCC 7002. (B) Colonies of *Synechococcus* sp. strain PCC 7002 transformed with *pcyA::aadA* on medium containing spectinomycin. Sectors and colonies with wild-type coloration are obvious. (C) PCR amplification of the *pcyA* locus from the wild type (lane 1) and the *pcyA::aadA* transformed strain (lane 2) of *Synechococcus* sp. strain PCC 7002. The two amplicons visible in lane 2 indicate that both the *pcyA* and *pcyA::aadA* alleles are present in the genomic DNA of the transformed cells.

PBPs (Fig. 2B). Despite continued careful selection of colonies lacking the dark, blue-green pigmentation of the wild type, colonies continued sectoring. This suggested that wild-type alleles of *pcyA* continued to persist in the transformed cells. PCR analyses confirmed the presence of both the *pcyA* and *pcyA::aadA* alleles within the population (Fig. 2C), and full segregation of the *pcyA* and *pcyA::aadA* alleles was never observed. These observations suggested that under standard growth conditions the *pcyA* gene of *Synechococcus* sp. strain PCC 7002 is essential.

Attachment of PEB to CpcA in *E. coli* and *Synechococcus* sp. strain PCC 7002. Heterologous production of holo-PCB-CpcA in *E. coli* has previously been demonstrated (43). Holo-PEB-CpcA can also be produced when *pebS* is provided as an alternative ferredoxin-dependent biliverdin reductase and coexpressed with *cpcA* and the *cpcE/cpcF* lyase in *E. coli*. This observation prompted a study to determine if this phenomenon could be replicated in a cyanobacterium, in which chromophory-

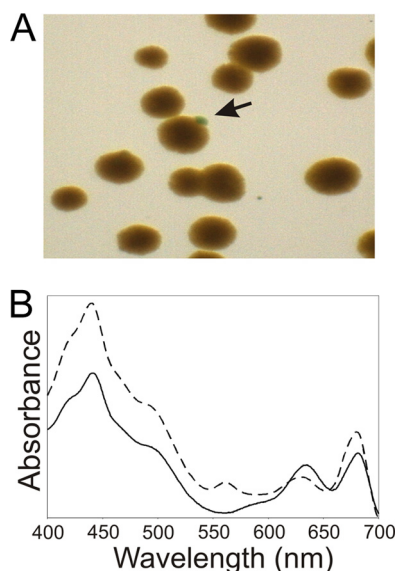


FIG. 3. Overexpression of *pebA* and *pebB* in *Synechococcus* sp. strain PCC 7002. (A) Colonies of *Synechococcus* sp. strain PCC 7002 transformed with the *pebAB* overexpression cassette mostly exhibit a reddish-brown phenotype resembling strains that naturally synthesize PE. A sector that has reverted to the wild-type color phenotype can be seen (arrow). (B) Whole-cell absorption spectra of wild-type *Synechococcus* sp. strain PCC 7002 (solid line) and the strain overexpressing *pebA* and *pebB* (dashed line).

lation might be more tightly controlled because of the potential to cause deleterious effects on the photosynthetic capacities of cells. For this experiment, *pebA* and *pebB*, encoding 15,16-dihydrobilibiverdin:ferredoxin oxidoreductase and phycoerythrobilin:ferredoxin oxidoreductase, respectively, from the chromatically acclimating cyanobacterium *Synechococcus* sp. strain PCC 7335 were placed under the control of the strong *cpcBA* promoter of *Synechocystis* sp. strain PCC 6803 and introduced into a neutral site on the endogenous pAQ1 plasmid of *Synechococcus* sp. strain PCC 7002 (47). This construct was transformed into a glycerol-tolerant strain, which was grown mixotrophically with glycerol. As for the transformants containing the *pcyA::aadA* construct, transformants possessing the *pebAB* construct were initially indistinguishable from the wild type. However, after several successive rounds of selection and streaking, colonies began to show regions with a brownish to brick red color. By careful selection of colonies, it was eventually possible to isolate nearly uniformly reddish-brown colonies that resembled the colonies of cyanobacterial strains that naturally produce PE. Despite numerous attempts to grow this strain under various green light intensities, which might favor cells with increased green absorption, complete elimination of colonies with the blue-green coloration of the wild type was never completely achieved (Fig. 3A). The absorption spectrum of a typical transformant strain (Fig. 3B) revealed an additional absorption peak at about 556 nm (the absorption maximum of PE) and an apparent decrease in the relative absorption by PC at ~635 nm.

To assess the PBP composition of cells overproducing PebA and PebB, intact PBS were isolated. In order to have sufficient cell material for analysis, liquid cultures were started with a

relatively high inoculum and were grown for a relatively short period (~40 h). Smaller starter inocula and/or longer incubation times tended to favor the appearance of revertants that resembled wild-type cells and lacked brown pigmentation. This might have been due to the accumulation of mutations that inactivated the expression of *pebA* and *pebB*, which would allow more rapidly growing, wild-type-like cells to predominate. Despite the instability of the genotype/phenotype, by keeping the inocula large and the incubation times minimal, it was possible to grow cells that retained most of the phenotype that was visible on plates (Fig. 3A).

In order to examine the distribution of PEB among the major PBPs isolated from PBS purified from the *pebA pebB* and wild-type strains, PBPs were separated by analytical SDS-PAGE alongside aliquots of purified recombinant His₆-CpcA carrying PCB or PEB. The proteins were detected by both zinc-enhanced fluorescence and by staining with Coomassie blue (Fig. 4A). Using excitation lasers at 532 nm and 633 nm, the zinc-enhanced fluorescence was imaged on a Typhoon 8600 variable mode imager. The results were compared to those for proteins with known chromophore contents to assess qualitatively which polypeptides carried PEB chromophores in the PBS isolated from cells overproducing PebA and PebB. CpcA was significantly more fluorescent when scanned with the 532-nm laser than was the case for CpcA from the wild-type strain, and the intensity of its fluorescence was similar to that of the recombinant His₆-CpcA-PEB protein standard. These same PBP samples were subsequently analyzed by HPLC in order to confirm these results and to determine if PEB was incorporated into any of the other PBS components. When the absorbance profiles of the major PBP components of PBS isolated from the *pebA pebB* overexpression strain (Fig. 4C) were compared to those from the wild-type strain (Fig. 4B), only CpcA exhibited a major difference in absorption. A very small amount of PEB was also bound to the ApcA subunit, but little or no PEB was incorporated into CpcB (i.e., the PC β -subunit). These results confirmed that PEB was mostly attached to CpcA and that very little PEB was incorporated into other PBP subunits that could be assembled into PBS.

***pcyA* could be inactivated in a strain expressing HY2.** When the *Arabidopsis thaliana* HY2 gene was introduced into plasmid pAQ1 of *Synechococcus* sp. strain PCC 7002 under the control of the very strong *cpcBA* promoter of *Synechocystis* sp. strain PCC 6803 (47), the transformed strain appeared slightly less blue, and thus slightly greener, than the WT (data not shown). An examination of the absorption spectra for this strain revealed that the absorption maximum for PC was red-shifted by approximately 10 nm (Fig. 5). Absorption shifts of a similar magnitude, about 10 nm to a shorter wavelength, have typically been observed when phytochromes have been reconstituted with PCB instead of P Φ B (15). Because of the very high expression levels of HY2 expected when driven by the *cpcBA* promoter, and the structural similarity of the resulting P Φ B chromophore to the native PCB chromophore, it seemed possible that the large proportion of the PBPs in these cells might carry P Φ B and not PCB chromophores. Previously, it was demonstrated that *pcyA* can largely complement a HY2-deficient strain of *A. thaliana*; this indicates that the two chromophores are nearly interchangeable in *A. thaliana* (24). Because this strain carried functional copies of both *pcyA* and

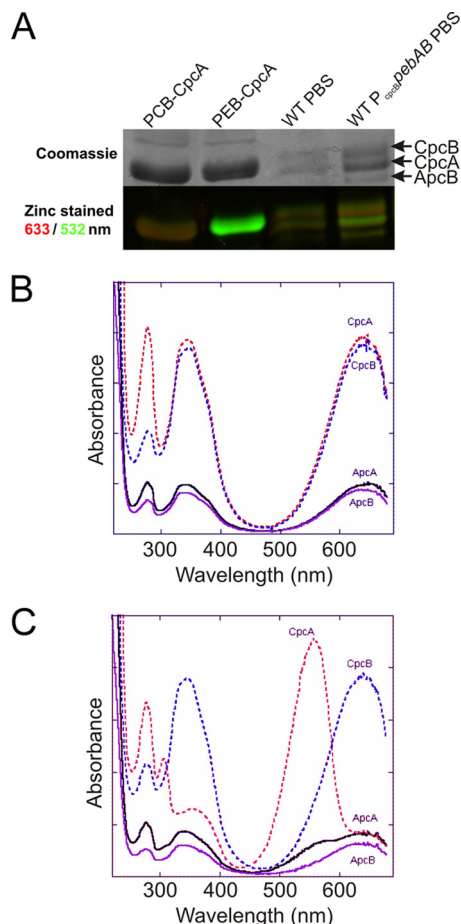


FIG. 4. Overexpression of *pebA* and *pebB* causes PEB to be ligated preferentially to CpcA. (A) Coomassie brilliant blue and zinc-enhanced fluorescence images of a single SDS-PAGE gel. Lane 1, recombinant CpcA carrying PCB; lane 2, recombinant CpcA carrying PEB; lane 3, PBS isolated from wild-type cells of *Synechococcus* sp. strain PCC 7002; lane 4, PBS isolated from cells of *Synechococcus* sp. strain PCC 7002 overexpressing *pebA* and *pebB*. (B) Absorption spectra of major PBP subunits separated by HPLC from PBS isolated from wild-type *Synechococcus* sp. strain PCC 7002. (C) Absorption spectra of major PBP subunits separated by HPLC from PBS isolated from cells of *Synechococcus* sp. strain PCC 7002 overexpressing *pebA* and *pebB*. Note that the CpcA subunit mostly carries a PEB chromophore with absorption at ~ 560 nm, although some absorption from PCB is also evident.

HY2, it was possible that sufficient PCB was available to allow the selective assembly of some PBPs with PCB, as was seen for the strain overexpressing *pebA* and *pebB*.

In order to determine if PFB could serve as the only bilin chromophore for *Synechococcus* sp. strain PCC 7002, cells overproducing HY2 were transformed with the same *pcyA::aadA* construct previously used unsuccessfully in the attempt to inactivate *pcyA* in the wild type. In contrast to the previous results, the transformant colonies in which *pcyA* was inactivated in the HY2 overexpression background did not develop a chlorotic appearance, and segregation of the mutant and wild-type alleles was rapidly achieved (Fig. 6B). The absorption spectrum of the resulting strain was similar to that of the background strain (Fig. 5 and 6A). However, because no wild-type alleles of *pcyA* could be detected by PCR, it was likely that

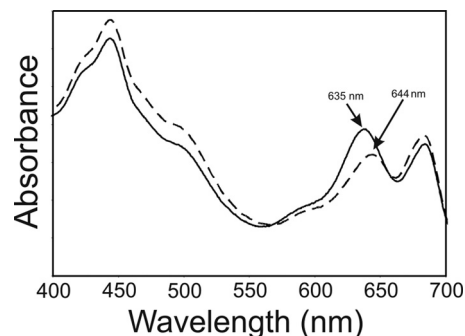


FIG. 5. Absorption spectra of *Synechococcus* sp. strain PCC 7002 expressing HY2. Absorption spectra of *Synechococcus* sp. strain PCC 7002 wild-type cells (solid line) and of a strain in which HY2 expression is being driven by the *cpcBA* promoter of *Synechocystis* sp. strain PCC 6803 (dashed line) are shown.

all of the PBPs produced in this strain carried PFB rather than PCB chromophores.

Properties of cells and PBS derived from a *pcyA* mutant expressing HY2. In order to examine the effects of global changes in PBS comprised of PBPs in which PCB was completely replaced by PFB chromophores, low-temperature (77 K) fluorescence emission spectra of cells were measured. Spectra for both the wild type and the strain overexpressing HY2 in a *pcyA* deletion background ($\Delta pcyA::aadA$ + HY2) were recorded with the excitation wavelength set to 590 nm, to excite mainly the PBPs (Fig. 7). For the wild type, the observed maxima at ~ 655 nm, ~ 670 nm, and ~ 688 nm represented emission from PC, AP, and the terminal emitters ApcD and

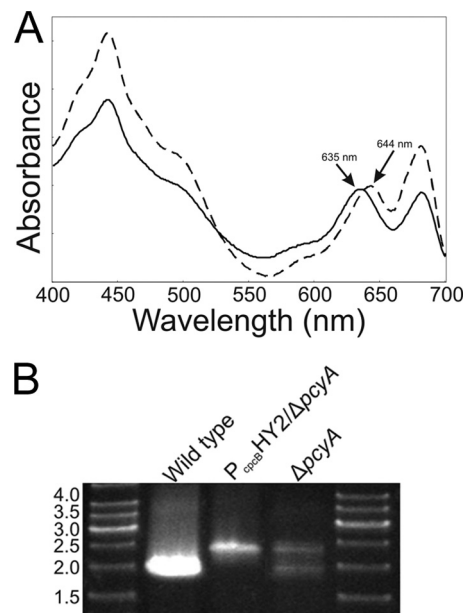


FIG. 6. HY2 functionally substitutes for *pcyA* in *Synechococcus* sp. strain PCC 7002. (A) Whole-cell absorption spectra of wild-type *Synechococcus* sp. strain PCC 7002 (solid line) and a strain overexpressing HY2 in which *pcyA* has been inactivated (dashed line). (B) PCR analysis of the *pcyA* locus in wild-type *Synechococcus* sp. strain PCC 7002 (lane 1), a strain overexpressing HY2 in which *pcyA* has been deleted (lane 2), and the *pcyA* partial deletion strain (lane 3).

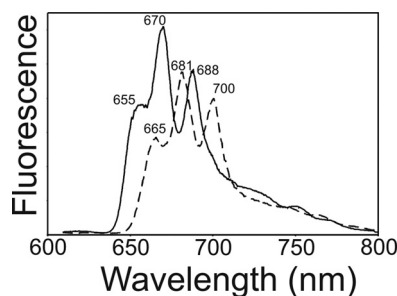


FIG. 7. The 77 K fluorescence emission spectra of *Synechococcus* sp. strain PCC 7002 wild-type cells (solid line) and cells of the *pcyA* deletion strain overexpressing HY2 ($\Delta pcyA::aadA$ + HY2 strain) (dashed line). The excitation wavelength was 590 nm.

ApcE, respectively (13, 37). As expected, based on the absorption profile of the $\Delta pcyA::aadA$ + HY2 strain, the replacement of all PCB chromophores with PΦB caused a red shift in the fluorescence emission of the PBPs in this strain. Fluorescence emission peaks for PC, AP, and ApcD/ApcE each shifted 12 to 14 nm, with the new maxima occurring at 665 nm, 681 nm, and 700 nm, respectively. Although the fluorescence intensity was lower for the mutant strain, the relative intensities of the three emission bands were similar for the mutant strain. This observation suggested that the relative amounts of PC, AP, and terminal emitters had not significantly changed in the PBS of the mutant strain.

The results described above for the $\Delta pcyA::aadA$ + HY2 strain were further corroborated by analyses of the PBS from the mutant strain (Fig. 8). Zinc and Coomassie staining of the gel showed generally similar chromophore contents and similar relative abundances of the PBS components in the two strains (Fig. 8A and B). Although similar amounts of the CpcA and ApcB proteins were loaded on the gel, as evidenced by the Coomassie staining, the CpcB band from the $\Delta pcyA::aadA$ + HY2 strain was much less focused and showed less signal when the same gel was zinc stained and visualized by fluorescence (Fig. 8A and B). This suggested that a portion of the CpcB subunit might be assembled into PBS with only a single PΦB chromophore. As expected, the absorbance and fluorescence emission maxima of the PBS isolated from the $\Delta pcyA::aadA$ + HY2 strain were red-shifted relative to those of wild-type PBS (Fig. 8C and D). Wild-type PBS had an absorption maximum at 630 nm, while PBS from the $\Delta pcyA::aadA$ + HY2 strain had an absorption maximum at 640 nm (Fig. 8C). The 77 K fluorescence emission of WT PBS exhibited maxima at 655 nm from PC and 683 nm from the terminal emitters ApcD and ApcE. The 77 K fluorescence emission spectrum of the PBS isolated from the $\Delta pcyA::aadA$ + HY2 strain had red-shifted emission maxima at 668 nm and 694 nm. When PBS samples of equal maximal absorption were compared, the PBS from the $\Delta pcyA::aadA$ + HY2 strain showed much less fluorescence emission from the terminal emitters. This result suggested that energy transfer to the terminal emitters was much less efficient in PBS produced from proteins carrying PΦB chromophores (Fig. 8D). Lastly, the absorption spectra of the PBS samples denatured in acidic urea were examined (Fig. 8E). As expected due to the larger number of conjugated double bonds in the PΦB chromophore, the absorption maximum of the denatured

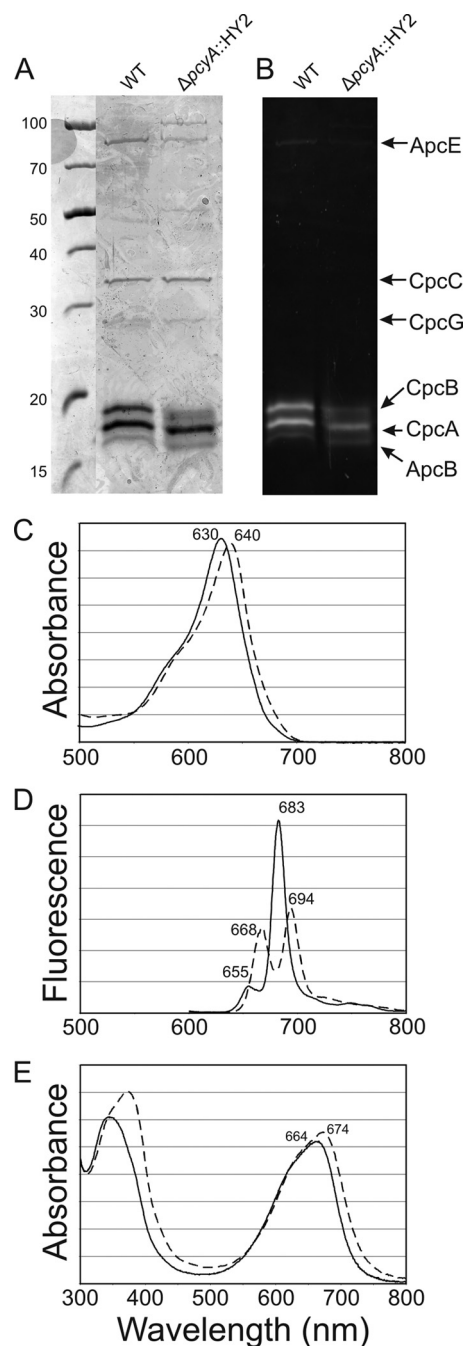


FIG. 8. Analysis of isolated PBS from the wild type and a $\Delta pcyA::aadA$ strain overproducing HY2. (A and B) SDS-PAGE results with isolated PBS from the wild-type and the $\Delta pcyA::aadA$ + HY2 strain (lanes labeled as $\Delta pcyA::HY2$), visualized by Coomassie staining (A) or by zinc-enhanced fluorescence (B). Selected proteins are identified by the arrows on the right. (C to E) Absorption spectra (C), 77 K fluorescence emission (D), and absorption spectra after denaturation in acidic urea (E) of PBS isolated from the wild type (solid lines) and a $\Delta pcyA::aadA$ + HY2 strain (dashed lines). The absorption spectra in panel C were normalized at the maximal values to facilitate the comparison. The fluorescence emission spectra were not normalized, and samples of equal absorption at 630 nm or 640 nm were used for the measurement. The excitation wavelength was 590 nm.

PBS from the $\Delta pcyA::aadA$ + HY2 strain was red-shifted ~ 10 nm, from 664 nm for the WT to 674 nm for the mutant strain.

Comparisons of the PBP and Chl contents of the wild type and the $\Delta pcyA::aadA$ + HY2 strain revealed that this strain contained about 40% less PBPs than the wild type, while the Chl contents of the two strains remained similar (data not shown). Taken together, the fluorescence emission properties of isolated PBS indicated not only that the PBPs are less abundant in the $\Delta pcyA::aadA$ + HY2 strain, but also that they are less effective in transferring absorbed light energy to the terminal acceptors of the PBS. Thus, the $\Delta pcyA::aadA$ + HY2 strain should exhibit growth defects due to impaired light harvesting.

The apparent requirement for either PCB or PFB chromophores by *Synechococcus* sp. strain PCC 7002 allowed for the cultivation and further characterization of this strain in the absence of the antibiotics normally used to select for strains harboring the recombinant DNA constructs. Growth rate measurements were conducted to determine the effects of the altered bilin content on the light-harvesting capabilities of this strain. Growth rates were determined under standard growth conditions for *Synechococcus* sp. strain PCC 7002 (38°C, 1% [vol/vol] CO₂ in air, nitrate as N-source) at three different light intensities: 50, 200, and 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 9). When light was limiting (50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), the doubling time for the $\Delta pcyA::aadA$ + HY2 strain was more than 2-fold longer than that of the wild type. At an intensity slightly less than saturating (200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), the doubling time for the $\Delta pcyA::aadA$ + HY2 strain was about 50% longer than the wild type. At a suprasaturating light intensity (500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), the growth rates for the two strains were virtually indistinguishable. These data showed that the $\Delta pcyA::aadA$ + HY2 strain was significantly impaired in light harvesting in comparison to the wild type but that the photochemical reaction centers were unaffected.

DISCUSSION

The inability to inactivate the *pcyA* gene in the genome of *Synechococcus* sp. strain PCC 7002 suggested that PcyA, and by extension its product, PCB, are normally required for cellular viability. This observation was surprising for several reasons. Previous studies had shown that it is possible to produce strains in which the genes for *cpcBA* and *apcAB* are deleted and the double mutant does not accumulate detectable levels of PBPs (9). In those studies, however, the apo-PBPs were not synthesized, and it remains a possibility that the additional back-selection pressure for retention of the capacity to synthesize the antennae prevented complete segregation of the *pcyA* and *pcyA::aadA* alleles in the experiments described here. An alternative explanation for these observations might be that there are other proteins that require PCB chromophores that are essential when cells are grown in continuous illumination. Candidates for such proteins include phytochrome and cyanobacteriochrome-like proteins encoded in the *Synechococcus* sp. strain PCC 7002 genome. While several of these are homologous to proteins characterized in *Synechocystis* sp. PCC 6803, neither their potential cognate chromophore nor the phenotype of mutants lacking the products of these genes has been investigated. The requirement for PCB for purposes other than the chro-

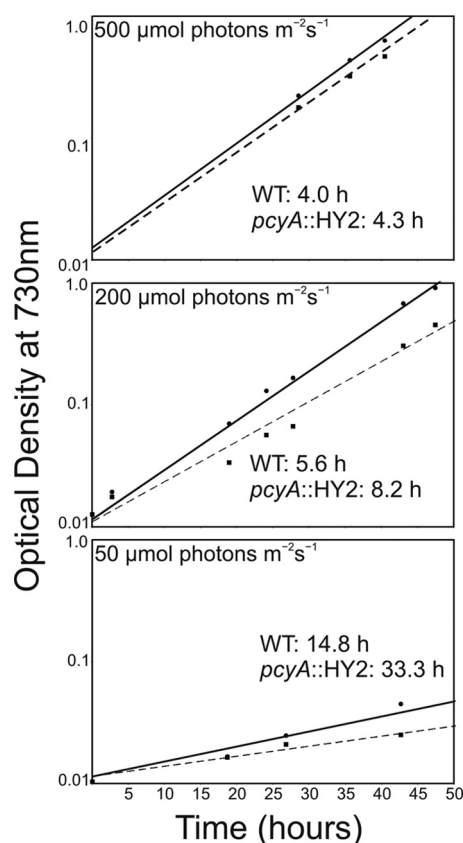


FIG. 9. Growth rate analysis of wild-type *Synechococcus* sp. strain PCC 7002 and the $\Delta pcyA::aadA$ + HY2 strain (labeled as *pcyA::HY2*). Cells were grown under otherwise standard conditions at high (supra-saturating; 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), medium (nearly saturating; 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), and low (limiting; 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) light intensities. The plotted data are the averages of results for at least three replicate cultures, and the calculated doubling times are indicated.

mophore for PBPs is further supported by the recent sequencing of the genome of the cyanobacterium UCYN-A, a marine cyanobacterium with a highly reduced genome (44). Although this genome no longer encodes any PBPs, it nevertheless has retained the *pcyA* gene and several genes for sensory proteins that may function as photoreceptors.

High-level expression of *pebAB* in the absence of PE apo-proteins or associated linker polypeptides produced cells with a whole-cell absorption spectrum that resembled that of a PE-producing cyanobacterium (Fig. 3). Strains with this phenotype were extremely unstable, however, and could only be maintained by carefully choosing the most reddish-brown colonies, and even with this precaution, apparent phenotypic revertants (to blue-green coloration) were common (Fig. 3A). When cells were cultivated for more than about 2 days, liquid cultures rapidly reverted to wild-type pigmentation and absorption. One explanation for this is that hyperexpression of *pebA* and *pebB* might limit the production of PCB. Because PcyA and PebA utilize the same substrate, biliverdin IX α , severe overexpression of *pebA* could limit the availability of PCB, which, as discussed above, appears to be required for viability when cells are grown in continuous light. Although

PCB in principle should still be produced, high levels of PEB might also competitively inhibit reactions in which PCB is the natural substrate. *In vitro* experiments using CpcA with its cognate CpcEF lyase have previously demonstrated that PEB can function as a competitive inhibitor for PCB addition (17). If PCB is required to produce a functional phytochrome-like molecule, PEB would probably be unable to satisfy this requirement, because it lacks the 15–16 double bond present in PCB that is the site of reversible photoisomerization (26).

The absorption phenotype of the strain overproducing *pebA* and *pebB* was almost exclusively due to the incorporation of PEB into CpcA, although a trace of PEB was also found on ApcA (Fig. 4). Whether this was due to the inability of the CpcS/CpcU and CpcT lyases to utilize PEB *in vivo* or to selective degradation of PBP subunits mischromophorylated with PEB was not determined in the studies reported here. Attempts to use the CpcS/CpcU and CpcT lyases to introduce PEB into other PBP subunits in heterologous expression systems have resulted in much lower levels of addition than those seen for CpcE/CpcF/CpcA (A. Biswas and W. M. Schlachter, unpublished observations). This suggests that PBP lyases other than CpcE/CpcF are capable of distinguishing PCB from PEB.

PΦB, the chromophore associated with phytochromes of plants, has a double bond at the 15–16 position and only differs from PCB by the presence of an additional double bond at the C-18 position. Because of the structural similarity to the native PCB, it was expected that overexpression of PΦB synthase (HY2) would be much less detrimental to the cell than overexpression of *pebA* and *pebB*. Expression of HY2 was sufficient to overcome the apparent growth defect that prohibits the inactivation of *pcyA* in a wild-type background, indicating that PΦB is an acceptable substitute chromophore for PCB. Except for the lower growth rate of this strain at limiting light intensities, the replacement of PCB by PΦB seemed to produce no additional deleterious effects. This was reflected by the fact that the mutant and wild-type strains had nearly identical growth rates when the strains were grown at high light intensity (Fig. 9). Although not directly addressed in this study, it is also important to note that light sensing is likely altered in this strain. Phytochrome-like photosensory proteins that normally utilize PCB or PVB should bind PΦB or phytyolobilin (the Δ5-to-Δ2 double-bond isomer of PΦB), although those that bind biliverdin are probably unaffected, because the cells can still synthesize biliverdin.

Previous studies on the PCs of red algae and marine *Synechococcus* sp. strains have shown that at least four alternative PCs occur (8, 38, 39). The red alga *Porphyridium cruentum* synthesizes an R-PC in which the β-155 Cys carries a PEB chromophore but the α-84 and β-84 positions carry PCB chromophores (22). In *Synechococcus* sp. WH8103, the β-84 position carries a PCB chromophore but the α-84 and β-155 positions carry PEB chromophores (31). The α-84 position of *Synechococcus* sp. WH8501 PC carries a PUB chromophore, while the two β-subunit cysteines carry PCB chromophores (42). Finally, there is a trichromatic type of R-PC found in *Synechococcus* sp. WH8102 that contains PUB on α-84, PEB at β-153, and PCB at β-82 (8). Interestingly, the configuration of one PEB on CpcA and two PCBs on CpcB, which occurs when *pebAB* is aberrantly expressed in *Synechococcus* sp. strain PCC 7002, is the same configuration reported to occur naturally on

R-PC-III of *Synechococcus* sp. WH7805 (30). PCs exhibit the highest diversity in chromophore contents among PBPs. Examples are known that can bind each of the four naturally occurring cyanobacterial chromophores: PCB, PEB, PVB, and PUB (38). The β-84 position of CpcB invariably carries PCB, however. These observations, as well as data showing that PE subunits can accommodate PUB chromophores in place of PEB chromophores in strains exhibiting type IV chromatic acclimation (16), demonstrate that at least some PBP subunits can readily accommodate alternative chromophores.

Given that cyanobacteria have evolved to use four different bilin chromophores for light harvesting and that a single amino acid substitution can cause PcyA to lose its ability to catalyze the reduction of the C-18 double bond and thus produce PΦB (45), it is surprising that no naturally occurring PBPs with PΦB chromophores have yet been found in cyanobacteria. The results reported here suggest two reasons why this might be the case. First, PBPs with PΦB chromophores have absorption properties that overlap more strongly with Chl *a*, so PBPs carrying PΦB chromophores produce less additional absorption cross-section (other than an increase in total chromophore number) for the two photosystems. Additionally, the intrinsic spectroscopic properties of PΦB may not be well suited for energy transfer. This is suggested by the lower apparent quantum yield of fluorescence and the apparently less-efficient energy coupling of major PBPs to the terminal emitters in PBS from the *pcyA* mutant strain overproducing HY2. Further characterization of purified PBPs carrying PΦB will be required to determine whether energy transfer is inefficient because of altered chromophore-protein interactions or whether some other property of PΦB limits the energy transfer efficiency of this chromophore.

In conclusion, this study shows that PBPs with unique properties can be generated rather easily by rather simple changes in the bilin biosynthetic pathway. Although it has long been recognized that gene duplication and divergence are important aspects of PBP diversification and evolution (1, 21, 38), an equally important component leading to the emergence of new light-absorbing molecules has been the duplication and divergence within the ferredoxin-dependent biliverdin reductase family (PcyA, PebS, PebA, PebB, and HY2) as well as the emergence of isomerizing bilin lyases (e.g., PecE/PecF). This study shows that modifications in bilin synthesis might be sufficiently well tolerated under some light conditions to allow genetic changes to occur that could ultimately be selected and fixed in populations. It is likely that bilin synthetases and bilin lyases have played very important roles in the evolution of proteins with new absorption properties. Thanks to recombinant methodologies, combinations of bilins and PBP not yet found or even possible in nature can be produced, characterized, and applied to the study of specific biological problems.

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